D E C L A R A T I O N

I, Ippei Andoh, 5-1-803, Takabashi, Koto-ku, Tokyo, Japan, do hereby declare that I am well acquainted with the Japanese language and English language and the attached English document is believed to be full, true, and faithful translation made by me of Japanese Patent Application 49636/1989. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Tokyo, Japan

This 29th day of November, 1991

I. Andoh

Ippei Andoh

PATENT OFFICE

JAPANESE GOVERNMENT

This is to certify that the annexed is a true copy of the following application as filed with this Office.

Date of Application: March 1, 1989

Application Number: 49636/1989

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March 23, 1990

Commissioner, Patent Office

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Certificate No. 13797/1990

Application for Patent

March 1, 1989

(14,000Yen)

To: Commissioner,
Patent Office
Fumitake Yoshida

1. Title of the Invention

NOVEL DNA FRAGMENT

2. Number of Inventions described in Claims: 5

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6. List of Appended Documents:

(1) Specification	one copy
(2) Duplicate copy of the Petition	one copy
(3) Power of Attorney	each one copy
(4) Drawings	one copy
(5) Copy of International Microorganism Deposition Receipt	one copy

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Specification

1. Title of the invention

A NOVEL DNA FRAGMENT

2. What is claimed is:

1. A DNA fragment comprising a base sequence encoding a polypeptide derived from human brain and possessing natriuretic activity.

2. The DNA fragment according to Claim 1, wherein said polypeptide has the following amino acid sequence:

His Pro Leu Gly Ser Pro Gly Ser Ala Ser Asp Leu Glu Thr Ser Gly Leu Gln Glu Gln Arg Asn His Leu Gln Gly Lys Leu Ser Glu Leu Gln Val Glu Gln Thr Ser Leu Glu Pro Leu Gln Glu Ser Pro Arg Pro Thr Gly Val Trp Lys Ser Arg Glu Val Ala Thr Glu Gly Ile Arg Gly His Arg Lys Met Val Leu Tyr Thr Leu Arg Ala Pro Arg Ser Pro Lys Met Val Gln Gly Ser Gly Cys Phe Gly Arg Lys Met Asp Arg Ile Ser Ser Ser Ser Gly Leu Gly Cys Lys Val Leu Arg Arg His

3. The DNA fragment according to Claim 1, wherein said polypeptide has the following amino acid sequence:

Met Asp Pro Gln Thr Ala Pro Ser Arg Ala Leu Leu Leu Leu Leu Phe Leu His Leu Ala Phe Leu Gly Gly Arg Ser His Pro Leu Gly Ser Pro Gly Ser Ala Ser Asp Leu Glu Thr Ser Gly Leu Gln Glu Gln Arg Asn His Leu Gln Gly Lys Leu Ser Glu Leu Gln Val Glu Gln Thr Ser Leu Glu Pro Leu Gln Glu Ser Pro Arg Pro Thr Gly Val Trp Lys Ser Arg Glu Val Ala Thr Glu Gly Ile Arg Gly His Arg Lys Met Val Leu Tyr Thr Leu

Arg Ala Pro Arg Ser Pro Lys Met Val Gln Gly Ser Gly Cys Phe Gly Arg Lys Met Asp Arg Ile Ser Ser Ser Ser Gly Leu Gly Cys Lys Val Leu Arg Arg His

4. The DNA fragment according to Claim 1 or 2 having the following base sequence:

CAC CCG CTG GGC AGC CCC GGT TCA GCC TCG GAC TTG GAA ACG TCC GGG TTA CAG GAG CAG CGC AAC CAT TTG CAG GGC AAA CTG TCG GAG CTG CAG GTG GAG CAG ACA TCC CTG GAG CCC CTC CAG GAG AGC CCC CGT CCC ACA GGT GTC TGG AAG TCC CGG GAG GTA GCC ACC GAG GGC ATC CGT GGG CAC CGC AAA ATG GTC CTC TAC ACC CTG CGG GCA CCA CGA AGC CCC AAG ATG GTG CAA GGG TCT GGC TGC TTT GGG AGG AAG ATG GAC CGG ATC AGC TCC TCC AGT GGC CTG GGC TGC AAA GTG CTG AGG CGG CAT

5. The DNA fragment according to Claim 1 or 3 having the following base sequence:

ATG GAT CCC CAG ACA GCA CCT TCC CGG GCG CTC CTG CTC CTG CTC TTC TTG CAT CTG GCT TTC CTG GGA GGT CGT TCC CAC CCG CTG GGC AGC CCC GGT TCA GCC TCG GAC TTG GAA ACG TCC GGG TTA CAG GAG CAG CGC AAC CAT TTG CAG GGC AAA CTG TCG GAG CTG CAG GTG GAG CAG ACA TCC CTG GAG CCC CTC CAG GAG AGC CCC CGT CCC ACA GGT GTC TGG AAG TCC CGG GAG GTA GCC ACC GAG GGC ATC CGT GGG CAC CGC AAA ATG GTC CTC TAC ACC CTG CGG GCA CCA CGA AGC CCC AAG ATG GTG CAA GGG TCT GGC TGC TTT GGG AGG AAG ATG GAC CGG ATC AGC TCC TCC AGT GGC CTG GGC TGC AAA GTG CTG AGG CGG CAT

3. Detail description of the invention

[Field of Industrial Application]

This invention relates to a novel DNA fragment useful for the production of physiologically active polypeptide, and more particularly to a DNA fragment possessing a base sequence encoding the polypeptide (human BNP) having natriuretic activity.

[Prior Art]

Structures of new polypeptides secreted by human or rat artium and having natriuretic activity have successively been determined and reported in the years 1983-1884 [*Biochem. Biophys. Res. Commun.* 117, 859 (1983); *Biochem. Biophys. Res. Commun.* 118, 131-139 (1984)]. These polypeptides were named artium natriuretic peptides (hereinafter referred to as "ANP"). Since they have strong natriuretic activity as well as relaxing activity of vessel and smooth muscle, they are attracting much attention as a new peptide medicine for circulation diseases.

In 1988, a new peptide having diuretic activity was isolated in a purified form from porcine brain. Its structure was determined and the peptide was named "porcine brain natriuretic peptide" (hereinafter referred to as porcine BNP or pBNP) [*Nature*, 332, No. 6159, 78-81 (1988); *Biochem. Biophys. Res. Commun.* 155, 726-732 (1988)]. Pharmaceutical activities of polypeptide having natriuretic activity (BNP) resemble those of ANP, and include diuretic activity, natriuretic activity, vasodepressor activity, chicken rectum relaxation activity, and the like. The specific activities of BNP also resemble those of ANP,

except that the rectum relaxation activity of BNP is 3 to 4 times higher than that of ANP. This is the reason that BNP is expected to be a new medicine for circulation disease and that studies involving DNA of porcine BNP are being undertaken. Cloning of cDNA possessing a base sequence encoding porcine BNP and its precursor has been reported [*Biochem. Biophys. Res. Commun.* 157 *(1)*, 410-416 (1988)].

[Problems to be solved by the Invention]

Since BNP is used for the treatment of human diseases, and from the aspect of antigenicity, the development of the human BNP has been desired . Such a peptide, however, has not been heretofore found. Nor has its structure been clarified neither on a DNA level nor on a peptide or protein level.

[Means to solve the Problems]

In view of this situation, the present inventors have conducted extensive studies to obtain human BNP, and have been successful in cloning cDNA encoding a BNP by screening a cDNA library of human tissues by using a cDNA fragment which encodes porcine BNP precursor.

Accordingly, an object of this invention is to provide a DNA fragment comprising a base sequence encoding a polypeptide derived from human brain and possessing natriuretic activity.

The DNA fragment of the present invention can be prepared, for example, by the following process.

The total RNA is separated from a human tissue which is

considered to contain human BNP. mRNA is isolated from the total RNA and a cDNA library is constructed by a conventional method. A DNA fragment encoding human BNP can be isolated by screening the cDNA library by means of hybridization with a probe which has DNA sequence encoding a porcine BNP precursor. The process is illustrated in more detail.

(1) Construction of cDNA library

The mRNA is prepared from tissue such as human brain, human artium, or the like. The RNA can be separated by homogenizing a human artium, for example, by homogenizing the artium together with guanidylthiocyanate, followed by equilibrated density gradient ultracentrifugation using cesium trifluoro acetate. The mRNA is purified according to a conventional manner using oligo (dT) cellulose column chromatography. Synthesis of cDNA from the mRNA is carried out according to a conventional method, e.g. the method using a cDNA synthesis kit (manufactured by Pharmacia Co.), the Okayama-Berg method, the method of Gubler, U. and Hoffman, B. J. or its modification, or a method using other commercially available kit. An endonuclease EcoRI adaptor is applied to cDNA thus obtained, following which the 5'-end is phosphorylated using T-4 polynucleotide kinase or the like. The cDNA library is then constructed by ligation using a vector, e.g. λgt10, followed by *in vitro* packaging.

(2) Screening of human BNP clone

Screening of human BNP clone is carried out using a

labeled porcine cDNA fragment as a probe. This cDNA fragment is, for example, a 120bp fragment which is obtained by the digestion of the incomplete clone obtained in the course of cDNA cloning of the porcine BNP using endonucleases XhoI and RsaI [*Biochem. Biophys. Res. Commun.* *157*, 410 (1988)]. The 120bp fragment comprises DNAs encoding the active portion of the porcine BNP (BNP-26) consisting of 26 amino acids and its upstream 30bp. A probe to be used is prepared by labeling this cDNA fragment with ^{32}P. The probe and the cDNA library prepared in (1) above are hybridized and the positive clone is selected. The DNA fragment of the present invention can be prepared by cleaving the selected positive clone λhBNP-57 with a suitable endonuclease.

To determine the base sequence of the DNA fragment thus obtained the following conventional method is used. For example, first preparing a phBNP-57 by incorporating the DNA fragment into a sequencing vector, then preparing a restriction endonuclease map of the cDNA region, and further incorporating DNA fragments produced by using endonucleases which can cut the DNA into a suitable length into sequencing vectors to obtain a subclone, and determining the whole base sequence by the method of Sanger et al.

The base sequence of the DNA fragment encoding human BNP thus determined and the amino acid sequence of the human BNP are shown in Figure 2. In the base sequence in Figure 2, the sequence 1-402 is considered to code for pre-pro-

human BNP which is comprised of 134 amino acids, and 79-402 of these bases are considered to code for pro-human BNP which is comprised of 108 amino acids. These supposition were based on the fact that the structure of the signal peptide located before the precursor is very similar to that of porcine, and that the porcine BNP also has the amino acid sequence Arg-Ser-His-Pro-Leu-Gly corresponding to the base numbers 73-90 and the Ser-His bond of this sequence is cut to form the porcine BNP precursor [*Biochem. Biophys. Res. Commun.* ***157***, 410 (1988)]. Among these, sequence 307-402 is considered to encode the human BNP-32 which is comprised of 32 amino acids. The precursor is considered to form the human BNP-32 as a result of the processing, although this is not decisive because the human BNP has never been successfully isolated as a peptide. Of porcine DNAs, porcine BNP-26 [*Nature*, ***332***, 78-81 (1988)] and porcine BNP-32 consisting of 32 amino acids [*Biochem. Biophys. Res. Commun.* ***155***, 726-732 (1988)] have been isolated. As for ANP, human αANP consisting of 28 amino acids from human tissue [*Biochem. Biophys. Res. Commun.* ***118***, 131-139 (1984)] and rat αANP consisting of 28 amino acids from rat tissue [*Biochem. Biophys. Res. Commun.* ***117***, 859-865 (1983)] have been isolated. All of them are produced by the processing which took place downstream of Arg and Pro-Arg existing in the precursor. In the case of human BNP, since an Arg preceding the peptide having 23 amino acids from Cys (112) to His (134) which is considered to exhibit activity is the

102 Arg and this Arg has a Pro-Arg structure, the human BNP-32 consisting of 32 amino acids is presumed to be produced as a result of the processing after the Pro-Arg structure.

Based on the fact that the ANP precursor had been found to have a physiological activity, the human BNP precursor is also considered to have some physiological activity. Thus, both the human BNP-32 and the precursor are useful as a medicine.

The whole DNA and the amino acid sequence deduced from the base sequence are as shown in Figure 2. Peptides which exhibit the activity is not necessarily limited to the whole peptide. For example, a peptide having a shortened C-terminal is useful. It is also possible to replace a portion of the DNA fragment by other codons encoding amino acids to produce a peptide having the activity as human BNP. The DNA sequence coding for these amino acids is not limited one specific sequence. Once the DNA fragment of the whole length is specified, a number of variant DNAs can be produced, and a peptide having activity can be produced using these variant DNAs. The natriuretic peptides of the present invention (human BNP), therefore, are not limited to the human BNP of the whole length, but include peptides having the BNP activity. Also, the base sequence encoding human BNP means those having the base sequence encoding such a peptide.

[Effects of the Invention]

A variety of peptides, including human BNP, pro-human BNP, and other peptides having a biological activity with them, can be prepared by using the DNA fragment of the present invention and introducing an expression vector according to a known method.

The human BNP produced by the present invention possesses excellent smooth muscle relaxation activity, diuretic or natriuretic activity, and vasodepressor activity. The BNP is safe as a medicine for human because it is derived from human, thus it can be used as a medicine for curing such diseases as cardiac edema, nephric edema, hepatic edema, pulmonary edema, hypertension, congestive heat failure, acute and chronic renal failure, and the like.

Any methods conventionally used for the administration of peptide medicines, e.g. intravenous injection, intramuscular injection, subcutaneous injection, sublingual administration, intracutaneous administration, rectum administration, or the like, can be employed for the administration of the peptide of the present invention.

A preferable dose, that can avoid dangerous or harmful side effects, is 0.5 μg/kg to 100 mg/kg.

[Examples]

The following description of the exemplary embodiments are given for illustration of the invention and are not intended to be limiting thereof.

Example 1

(1) Construction of cDNA library

Human artium (3 g) was pulverized by a treatment with liquid nitrogen. To this artium was added, according to the method of Chirgwin et al. [Chirgwin, J. W. et al. *Biochemistry*, *18*, 5294-5299 (1979)], a guanidiumthiocyanate aqueous solution, and the mixture was homogenized. The whole RNA was separated by equilibrium density gradient ultracentrifugation using cesium trifluoro acetate, following which the RNA was purified, according to a conventional method, by oligo (dT) cellulose column chromatography to isolate 37 μg of poly(A)$^+$RNA(mRNA).

cDNA was synthesized from 3 μg of mRNA by using a cDNA synthesis kit (product of Pharmacia Co.). After the addition of an EcoRI adaptor, the 5'-end was phosphorylated with T4 polynucleotide kinase and ligation was carried out using λgt10 as a vector. λgt10 arms which was digested by EcoRI and dephosphorylated was employed as a vector. For 2 μg of the λgt10, the amount of cDNA used for the ligation was 0.1 μg converted to the amount of RNA used for the cDNA synthesis. After the ligation, the product was packed using a packaging kit (Gigapack Gold, product of Stratgene Co.), and cDNA library was obtained.

A small amount of cDNA was inoculated into *E. coli* c600 and c600hfl in order to investigate the completeness of the cDNA library. As a result, it was found that among the plaque produced by *E. coli* c600, 90% was transparent and 10%

was turbid, evidencing that the share of the recombinant phage in the library was 90%. Furthermore, the number of plaques in the whole cDNA library was found to be 7×10^7.

(2) Screening of human BNP clone

Screening was carried out on 5×10^5 plaques expressed by inoculating a portion of cDNA library into *E. coli* c600hfl. The cDNA fragment encoding porcine BNP, which was labeled with ^{32}P, was used as a probe for the screening. The cDNA fragment was prepared from plasmid BNP-82 (320 bp) which is an incomplete clone obtained in the course of cDNA cloning of the porcine BNP [*Biochem. Biophys. Res. Commun.* *157*, 410 (1988)], and plasmid BNP-82 was digested by restriction endonucleases XhoI and RsaI to obtain a 120 bp fragment which consists of a DNA fragment encoding porcine BNP-26 and its upstream 30 bp. The 120 bp fragment was then purified by acrylamide gel electrophoresis.

Plaques were first transferred to a nylon filter, and neutralized after alkali treatment, following which DNA was fixed by UV irradiation. The filter was immersed into a 5 x Denhardts solution and a 4 x SSC solution of 0.6 M NaCl and 0.06 M sodium citrate containing 100 μg/ml of denatured salmon sperm DNA and 0.1% SDS at 60°C for 3 hours, thus effecting hybridization. A probe labeled with ^{32}P by a Random primed DNA labeling kit (Product of Belinger Manheim Co.) was added to the hybridization solution having the same composition as the prehybridization solution to a concentration of 2×10^6 cpm/ml, wherein the filter was

incubated overnight at 60°C.

The filter was then washed with a 2 x SCC solution containing 0.1% SDS, dried in the air, and subjected to autoradiography.

Fifty five (55) hybridization positive plaques were thus obtained. The 55 positive plaques were submitted to a test to detect whether they could hybridize using the DNA (680 bp) encoding human ANP as a probe, and were found that all were negative. This is an evidence that this cDNA is different from the known cDNA encoding human ANP. The above 55 positive plaques were monocloned and λ-phage DNA was prepared according to a conventional method. A DNA fragment obtained by cleaving the λ-phage DNA with the restriction endonuclease EcoRI was investigated and was found that a cDNA having a maximum length of about 700 bp was inserted into a clone which was named λhBNP-57. This inserted cDNA was incorporated into Blue Script (KS (+)) (product of Stratgene Corp.) which is a sequencing vector, thus producing phBNP-57. The *E. coli* containing this plasmid was named *E. coli* HB101/phBNP-57 and deposited with Fermentation Research Institute, Agency of Industrial Science and Technology (Deposition No. 2299, FERM BP-2299). A restriction endonuclease map of the cDNA region was prepared. cDNA fragments were prepared using suitable restriction endonucleases which could cut the cDNA into a suitable length and these fragments were again incorporated into the blue script and subcloned.

Figure 1 shows the restriction endonuclease cleaving sites of the cDNA region and the base sequence determination strategy. The base sequence was determined by the method of Sanger et al. [*Proc. Natl. Acad. Sci.*, USA, 74, 5463-5467 (1977)].

Figure 2 shows the cDNA base sequence and the amino acid sequence corresponding to the base sequence.

The inserted DNA sequence has a long translational region starting from a translation initiation codon, ATG, and ending a translation termination codon, TAA. The cDNA having the whole length of 692 bp is considered to have a 5'-side non-translational region of the base pair number -99 to -1, 1-78 codes for a signal peptide, and 79-402 codes for the human BNP precursor. Among these, 307-402 are considered to codes for the human BNP-32 consisting of 32 amino acids and 403-593 are a non-translational region.

The amino acid sequence corresponding to the base sequence of 307-402 encoding the human BNP-32 has a cyclic structure which is formed with the cysteine disulfide bond of 17 amino acids and is very similar to that of porcine BNP.

Example 2

A γ-BNP-producing vector can be obtained by using phBNP-57 clone, a recombinant plasmid which is constructed by insertion of a cDNA obtained by digestion of λhBNP-57 with the restriction endonuclease EcoRI into a plasmid blue script. More specifically, a new restriction endonuclease

recognition site and a translation initiation codon (ATG) can be introduced at the site immediately preceding the γ-BNP-code region of phBNP-57 by site-directional mutation. A fragment is isolated by the utilization of this new recognition site. The above fragment is then inserted into the expression vector at immediately downstream of the plasmid promoter, and the plasmid is inserted into *E. coli*. The *E. coli* is cultured with nutrients sufficient to synthesize polypeptide, followed by which γ-BNP is collected. Another method of obtaining the human BNP-32 or the fragment to be cultured by *E. coli* is changing the site of site-directional mutation and the base sequence on the inserted cDNA of phBNP-57. The human BNP-32 or the fragment is then inserted into the expression vector and the vector is introduced into *E. coli*. Thus the human BNP-32 or the human BNP is obtained from the cultured *E. coli*.

4. Brief description of the drawings

Figure 1 shows the base sequence determination strategy of cDNA fragment inserted into positive clone λhBNP-57 and the restriction endonuclease map of the cDNA.

Figure 2 shows the cDNA base sequence and the amino acid sequence deduced from the base sequence which was determined by the strategy shown in Figure 1.

... Concluded ...

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FIG. 1

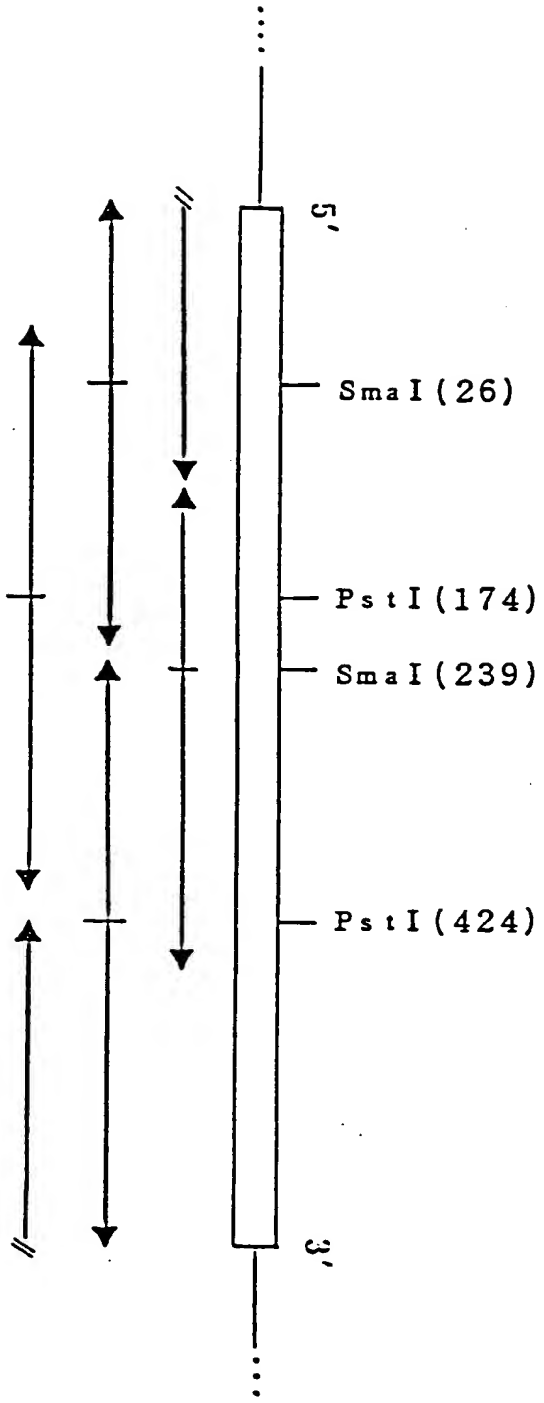
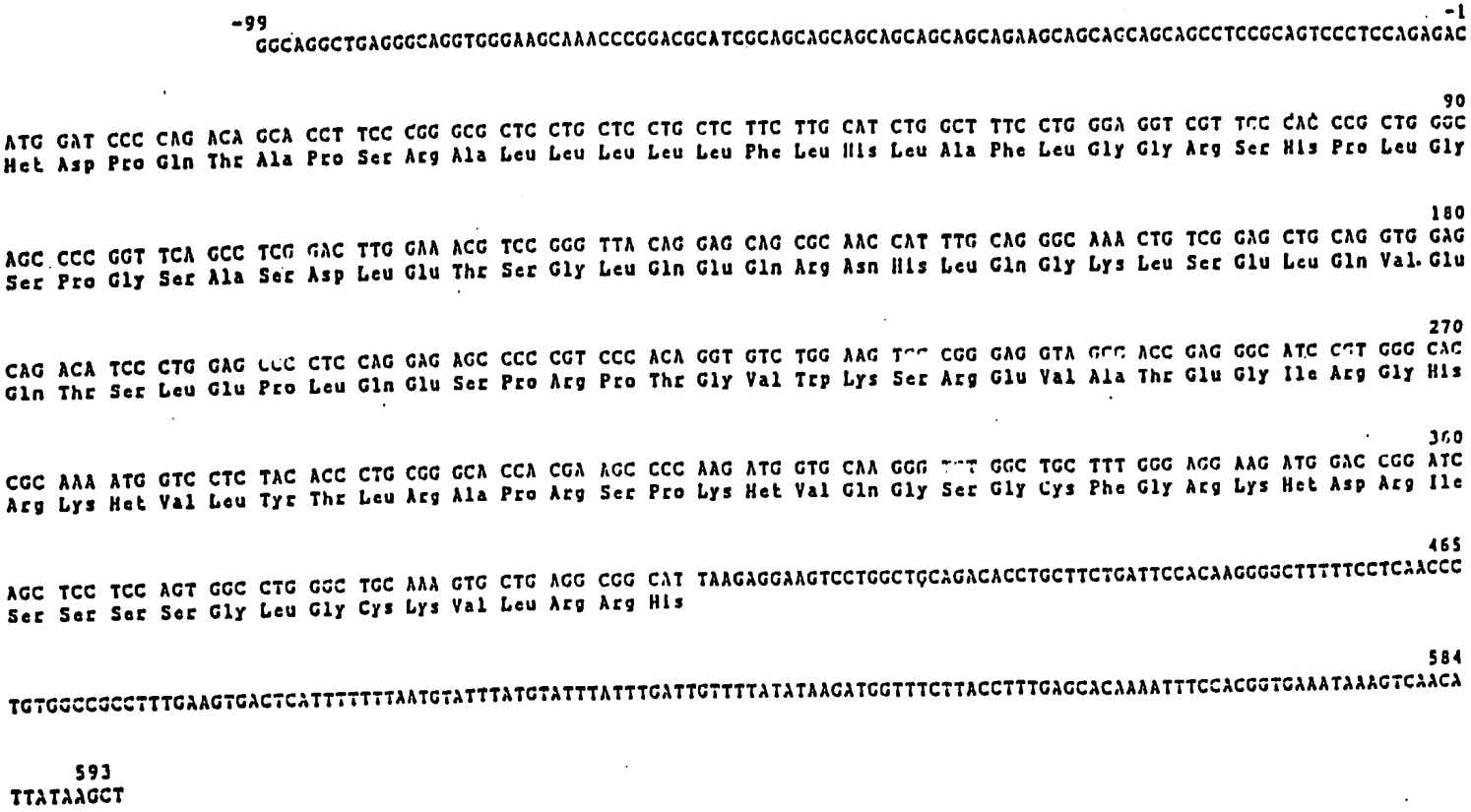


FIG. 2


-99
-1
GGCAGGCTGAGGGCAGGTGGGAAGCAAACCCGGACGCATCGCAGCAGCAGCAGCAGCAGAAGCAGCACGCAGCCTCCGCAGTCCCTCCAGAGAC
90
ATG GAT CCC CAG ACA GCA CCT TCC CGG GCG CTC CTG CTC CTG CTC TTC TTG CAT CTG GCT TTC CTG GGA GGT CGT TCC CAC CCG CTG GGC
Met Asp Pro Gln Thr Ala Pro Ser Arg Ala Leu Leu Leu Leu Leu Phe Leu His Leu Ala Phe Leu Gly Gly Arg Ser His Pro Leu Gly
180
AGC CCC GGT TCA GCC TCG GAC TTG GAA ACG TCC GGG TTA CAG GAG CAG CGC AAC CAT TTG CAG GGC AAA CTG TCG GAG CTG CAG GTG GAG
Ser Pro Gly Ser Ala Ser Asp Leu Glu Thr Ser Gly Leu Gln Glu Gln Arg Asn His Leu Gln Gly Lys Leu Ser Glu Leu Gln Val Glu
270
CAG ACA TCC CTG GAG CCC CTC CAG GAG AGC CCC CGT CCC ACA GGT GTC TGG AAG TCC CGG GAG GTA GCC ACC GAG GGC ATC CGT GGG CAC
Gln Thr Ser Leu Glu Pro Leu Gln Glu Ser Pro Arg Pro Thr Gly Val Trp Lys Ser Arg Glu Val Ala Thr Glu Gly Ile Arg Gly His
360
CGC AAA ATG GTC CTC TAC ACC CTG CGG GCA CCA CGA AGC CCC AAG ATG GTG CAA GGG TCT GGC TGC TTT GGG AGG AAG ATG GAC CGG ATC
Arg Lys Met Val Leu Tyr Thr Leu Arg Ala Pro Arg Ser Pro Lys Met Val Gln Gly Ser Gly Cys Phe Gly Arg Lys Met Asp Arg Ile
465
AGC TCC TCC AGT GGC CTG GGC TGC AAA GTG CTG AGG CGG CAT TAAGAGGAAGTCCTGGCTGCAGACACCTGCTTCTGATTCCACAAGGGGCTTTTTCCTCAACCC
Ser Ser Ser Ser Gly Leu Gly Cys Lys Val Leu Arg Arg His
584
TGTGGCCGCCTTTGAAGTGACTCATTTTTTTAATGTATTTATGTATTTATTTGATTGTTTTATATAAGATGGTTTCTTACCTTTGAGCACAAAATTTCCACGGTGAAATAAAGTCAACA
593
TTATAAGCT

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To: Daiich Pharmaceutical Co., Ltd.
Tadashi Suzuki, president

3-14-10, Nihonbashi, Chuo-ku,
Tokyo, 103 Japan

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR Escherichia coli HB101/phBNP57	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: FERM BP-2299
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: ☐ a scientific description ☐ a proposed taxonomic designation	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on February 21, 1989 (date of original deposit).	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Fermentation Research Institute **Agency of Industrial Science and Technology** **Tomoo Suzuki, DIRECTOR GENERAL** **1-3, Higashi, 1 chome, Tsukuba-shi,** **Ibaraki-ken 305, JAPAN** **February 21, 1989**	